DYNAMICS IN CORTISOL LEVELS IN *DANIO RERIO* FISH UNDER THE INFLUENCE OF A SYNTHETIC ANALOG OF KISSPEPTIN 1

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The effect of a synthetic analog of kisspeptin 1, a peptide involved in the regulation of the hypothalamic-pituitary-gonadal (HPG) stress axis, on the cortisol level of *Danio rerio* fish was investigated. Kisspeptin 1 was administered at doses of 2 μg/kg and 8 μg/kg followed by resting for 1 h and 4 h. We found that kisspeptin at doses of 2 μg/kg and 8 μg/kg increased cortisol levels, with a significant spike in cortisol levels at 1 h post-injection.

Key words: kisspeptin1; *Danio rerio*; cortisol; hypothalamic-pituitary-adrenal axis

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INTRODUCTION

Kisspeptin is a peptide that plays an essential role in the regulation of the hypothalamic-pituitary-gonadal (HPG) axis. Most of the neurons expressing kisspeptin are located in the rostral periventricular region of the third ventricle (RP3V) and the arcuate nucleus (ARC) of the hypothalamus [1]. However, kisspeptin neurons have projections to other brain regions, including the periventricular nucleus (AVPV) of the hypothalamus [2], thus suggesting a kisspeptin effect on the hypothalamic-pituitary-adrenal (HPA) axis [3]. The projection of corticotropin-releasing hormone (CRH) neurons onto kisspeptin neurons has also been reported [4]. In one of the first works related to the effect of HPA on kisspeptin it has been suggested that chronic or acute stress could lead to a decrease in kisspeptin; this would explain the suppression of luteinizing hormone (LH) production [5].

Kisspeptin is currently being considered as a potential pharmacological agent in the fight against reproductive system diseases, such as hypoganadotropic hypogonadism, polycystic ovary syndrome, and others. There are also reasons to consider its anxiogenic or anxiolytic effects.

The nucleotide sequences of kisspeptin-encoding genes are evolutionarily conserved and are observed in many vertebrates, including mammals. In *Danio rerio* (zebrafish) (Hamilton, 1822), two paralogous kisspeptin genes, *kiss1* and *kiss2*, have been identified; the former (kiss1) is the ortholog of the single human kisspeptin gene [6]. In *D. rerio*, kiss1 mRNAs have been found in the ventral region of the habenula, considered homologous to the lateral region of the habenula in mammals [7]. It has been shown that in both primates and *D. rerio* the habenula is involved in the neuromodulation of dopamine and serotonin and is thus associated with the stress response [8, 9] and, consequently, with the HPA axis.

The precursor of this kisspeptin (prepro-kisspeptin) is a 145 amino acid peptide, which is proteolytically cleaved into isoforms consisting of 54 residues, 14 residues, 13 residues, and 10 residues. The shortest peptides demonstrate the highest affinity for the receptor [10].

The length of the synthetic analog of kisspeptin corresponds to the isoform of 13 residues. Its posttranslational modifications are similar to the corresponding molecular transformations of human kisspeptin. This compound obtained by solid-phase peptide synthesis mimics the natural functional peptide in *D. rerio*. Hereafter, the analog of kisspeptin 1 will be referred to as the synthetic isoform consisting of 13 residues.

The aim of this study was to investigate the dynamics of cortisol changes as a marker of HPA activity on the action of a synthetic analog of kisspeptin 1 in zebrafish (*D. rerio*).

MATERIALS AND METHODS

The synthetic analog of kisspeptin 1 was obtained by solid-phase peptide synthesis using protective Fmoc-groups [11]. The purity of the resultant peptide preparation is more than 98%. Six groups of zebrafish (*D. rerio*) participated in the study.

Four experimental groups were injected with 1 μl of the peptide, while animals of the control groups were injected with 1 μl of 0.9% NaCl solution. The dose and duration of subsequent exposure are given in the Table 1.

Anesthesia and fixation of zebrafish were performed using 6% lidocaine (Synthesis, Russia), which was diluted to the final concentration of 40 mg/l. Zebrafish were placed in this solution for 5–6 min [12], then fixed and injected 1 μl of kisspeptin solution

Table 1. Characteristics of zebrafish groups and corresponding modes of interventions

Groups		Number of fish per group	Injections and time of exposure
G0	G0.1	12	0.9% NaCl, 1 h
	G0.4	12	0.9% NaCl, 4 h
G2	G2.1	8	Synthetic analog of kisspeptin 1, 2 μg/kg, 1 h
	G2.4	6	Synthetic analog of kisspeptin 1, 2 μg/kg, 4 h
G8	G8.1	8	Synthetic analog of kisspeptin 1, 8 μg/kg, 1 h
	G8.4	7	Synthetic analog of kisspeptin 1, 8 μg/kg, 4 h

intracerebrally with a syringe, then left in a tank with standard aquarium water (pH within 6.8–8.5, temperature 21–22°C, nitrates — less than 200 mg/l, carbon dioxide of not more than 20 mg/l, water hardness of 75–200 mg/l, no nitrites, dissolved oxygen of not less than 4 mg/l) for 1 h or 4 h. At the end of the exposures, zebrafish were removed from the experiment and tail muscle was sampled.

Homogenization of Samples

Cryogenic homogenization of the tail muscle tissue was performed using a Cryomil cryogenic vibration mill (Retsch, Germany) at -198°C for 8 min at 25 s^{-1}. Cooling of the biomaterial was carried out with liquid nitrogen supply. The resultant tissue homogenate was diluted in 620 μl of potassium-sodium-phosphate buffer, containing 136 mM sodium chloride, 2 mM potassium chloride, 10 mM phosphate buffer, pH 7.4.

Enzyme-Linked Immunosorbent Assay (ELISA)

The ELISA test system Alkor Bio (Russia) was used for determination of cortisol content. Optical density was measured at 450 nm using a Reader Synergy 2 photometer (Biotek, USA). Total protein content was measured using the Bradford method. Cortisol content per total protein was determined using the Elisa calculator software package (arigo Biolaboratories Corp., Taiwan).

Statistical Processing of Data

Statistical data processing was performed using GraphPad Prism 8.0. Normality of distribution was checked by the Kolmogorov-Smirnov criterion. Further processing to identify significant differences between experimental and control groups was performed using the Mann-Whitney and Kruskal-Wallis criteria with Dunn's multiple comparisons criterion. Statistical decisions were made at 5% significance level. Data are presented as median with interquartile range (Q1-Q3).

RESULTS

We found a statistically significant increase in cortisol levels in the experimental groups compared with the G0.1 control group (Fig. 1). However, no differences were found between the experimental groups.

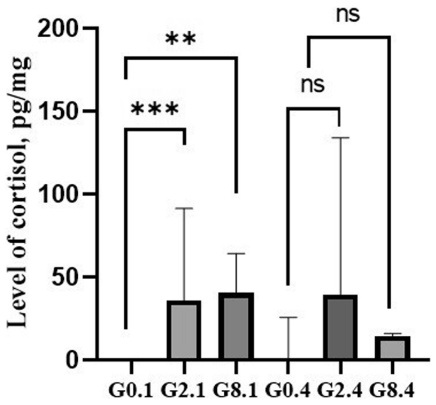

Figure 1. The cortisol level in the tail muscle of *D. rerio* 1 h and 4 h after injection of a synthetic analog of kisspeptin 1 as a function of the dose of the injected substance, pg/mg total protein, median and the interquartile range by Q3. **, $p<0.01$ ***, $p<0.001$.

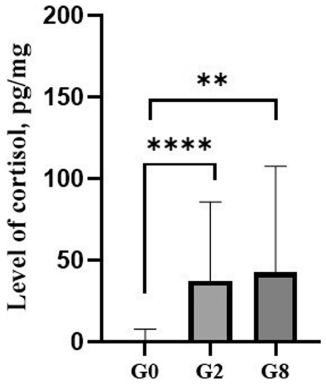

Figure 2. The maximal cortisol level in the tail muscle of *D. rerio* as a function of the dose of injected kisspeptin analog, pg/mg total protein, median and the interquartile range by Q3. **, $p<0.01$, ****, $p<0.0001$.

No statistically significant differences were found between groups of zebrafish that were left at rest for 4 h after injection (Fig. 1).

Comparing the changes in cortisol levels at different doses of kisspeptin 1 regardless of the subsequent resting time, we found an increase (Fig. 2) in cortisol secretion in groups G2 and G8 relative to the control group.

Comparison of zebrafish groups with different resting times (but the same dose of injected kisspeptin 1) did not reveal any statistically significant changes in cortisol levels. Consequently, when kisspeptin 1 is injected, the maximal recorded cortisol level in the tail muscle of *D. rerio* is independent of the subsequent time exposure.

Thus, it can be concluded that kisspeptin 1 injections at doses of 2 μg/kg and 8 μg/kg are accompanied by the increase in the cortisol level in the tail muscle of *D. rerio* relative to the control. More intense cortisol secretion occurs in the first hour after injection, whereas 4 h after kisspeptin 1 injection probably does not contribute to the change in stress hormone levels. We have shown that the time factor at the same drug dose also does not contribute to the change in cortisol levels.

DISCUSSION

Our results indicate that administration of both doses of the kisspeptin 1 analog increases cortisol levels. A significant effect of the drug is evident 1 h after injection and the cortisol level increases with the dose of the drug. No effect of kisspeptin 1 was observed 4 h after injection. Thus, in our study we observe the effect of synthetic analog of kisspeptin 1 on cortisol release.

The stress axis stimulating effect of kisspeptin was also demonstrated by Delmas et al. on male mice with knockout of the kisspeptin receptor gene [13]. Increased corticosterone secretion was also observed during intracerebrovascular administration of kisspeptin 8 and kisspeptin 13 in rats [14, 15]. Contrary evidence also exists that stress in its various variations (immune, psychosocial, etc.) contributes to a decrease in hypothalamic kisspeptin 1 mRNA in rats [16].

However, the addition of the kisspeptin 1 analog to water has been shown to reduce anxiety in *D. rerio* [17]. In this case, it is likely that the differences can be explained by the way of drug administration and its proportion of hits to the target. Thus, the available literature data confirm our conclusion about the pharmacological action of kisspeptin by intracerebral administration. Based on the results published by Goltz et al. it is reasonable to suggest that reduced levels of stress hormone can be achieved with lower dose injections.

During discussion of the action of kisspeptin, the possibility of a hormetic response of the stress axis was also mentioned [16]; this indirectly supports the assumption of a variable effect of the neuropeptide depending on its dose. The results of our study on zebrafish are consistent with those in mammals, but the mechanism of interaction between kisspeptin 1 and HPA should be further studied in more detail.

CONCLUSIONS

Thus, administration of a synthetic analog of kisspeptin 1 at doses of 2 μg/kg and 8 μg/kg results in an increase in cortisol in the *D. rerio* tail muscle with a significant increase in the level of this hormone occurring 1 h after injection.

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COMPLIANCE WITH ETHICAL STANDARDS

All manipulations with animals were approved by the Local Ethical Committee of the Institute of Experimental Medicine (IEM) (protocol No. 12 of September 26, 2019).

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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ДИНАМИКА КОРТИЗОЛА У РЫБ *DANIO RERIO* ПОД ВОЗДЕЙСТВИЕМ СИНТЕТИЧЕСКОГО АНАЛОГА КИССПЕПТИНА 1

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Исследовали воздействие синтетического аналога кисспептина 1 — пептида, участвующего в регуляции гипоталамо-гипофизарно-гонадной (ГГГ) стрессовой оси, — на уровень кортизола рыб *Danio rerio*. Кисспептин 1 вводили в дозах 2 мкг/кг и 8 мкг/кг с последующим покоем в течение 1 ч и 4 ч. Мы выяснили, что кисспептин в дозах 2 мкг/кг и 8 мкг/кг способствует повышению уровня кортизола, и значительный его всплеск приходится на 1 ч после инъекции.

Полный текст статьи на русском языке доступен на сайте журнала (http://pbmc.ibmc.msk.ru).

Ключевые слова: кисспептин 1; *Danio rerio*; кортизол; гипоталамо-гипофизарно-надпочечниковая ось

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